The Vitamin B_{12} Activity of Natural Material with Particular Reference to Cod Liver Protein

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ABSTRACT

Investigations concerning the stability of a group of factors contained in cod liver residue and displaying vitamin B_{12} activity have led to the conclusion that under the conditions prevailing during the rendering process, as developed at the Atlantic Fisheries Experimental Station, little or no deactivation occurs. This is in sharp contrast with what occurs under more drastic conditions such as used in the alkali-digestion process.

The study of the microbiological method used for the determination of vitamin B_{12} activity has shown that one step of the analytical procedure, namely, that phase during which the extraction of the sample is performed, is responsible for anomalous results. Deactivation occurs during this stage to an extent which varies according to the previous history of the material. The addition of thioglycolic acid or ascorbic acid during this period does not effectively protect the material unless culture medium is added as well. Medium alone is inefficient. Sodium bisulphite on the other hand exerts protection even in the absence of medium. This effect of sodium bisulphite applies not only to cod liver residue, but also to materials of diverse origin, mammalian viscera included. The data obtained show that vitamin B_{12} itself is not involved; presumably vitamin B_{12a} and vitamin B_{12b} are, although there is no direct evidence of their presence in cod liver.

As a protection during extraction, 0.08 per cent to 0.1 per cent of sodium bisulphite calculated on the amount of wet material appeared to be optimum.

INTRODUCTION AND METHODS

Cod liver solids have been made available in quantity by a new process [9] for the production of cod liver oil. In contrast to the processes hitherto in use, the livers are not subjected to undue exposures to high temperature or alkalinity. It was expected that the residue from this operation would still contain sensitive growth factors which display vitamin B_{12} activity and are originally present in appreciable amounts.

The identities of these factors are not known. Consequently, reference is made throughout the present paper to "factors" for which a "vitamin B_{12} activity" is determined by comparing a total growth-promoting effect on *L. leichmannii* with that produced on the same organism by known amounts of pure vitamin B_{12}.

Strong alkalinity and heat, the conditions prevailing during the course of the widely used alkali-digestion process, destroy these factors to a very large extent. In view of the economic importance of the latter, it seemed pertinent to evaluate the advantages of the new rendering process by studying the variations in vitamin B_{12} activity of the liver residue under the influence of heat over a wide range of pH values.

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The microbiological method used for the determination of vitamin B_{12} activity of our liver material gave very reproducible results. It became apparent, however, that they could not be trusted blindly. While in most cases they truly represented the degree of destruction induced by heat and alkali, in others loss in activity was indicated when no destruction had occurred.

Various experiments were carried out with the purpose of correcting this anomaly. Among them, our attempts to protect the factors from possible degradation during the analysis led to the finding of an interesting property of sodium bisulphite. This reagent produced a considerable increase of activity when added to the sample prior to autoclaving (extraction).

It was demonstrated that sodium bisulphite by itself has no growth-promoting activity. Maximum effect is attained when it is used at an optimum concentration. Assays made on materials other than cod liver residue show this effect of sodium bisulphite to be of general application.

Analytical Method

(*a*) preparation of samples. The sample submitted to the microbiological analysis was always an aqueous extract of the original material. The latter was usually a paste: cod liver residue mostly, or some other fish liver residue, or some natural tissue of a different origin. In any case, before applying the extraction procedure described below, it was found necessary, in order to ensure a good extraction, to remove the fat or residual oil from these pastes after they had been homogenized in a Waring Blendor. This defatting was accomplished as follows: the homogenized material was frozen at −10°C. Thin slices totalling about 10 g. were cut from the frozen substance, weighed in a large, wide-mouthed weighing bottle and repeatedly extracted with petroleum ether. The content of the bottle was then dried over phosphorus pentoxide.

The weighed dried residue was extracted in the following way. It was ground in a mortar with a little distilled water, suspended in water to a total volume of 50 ml.; the pH was adjusted to 5.5 and the mixture autoclaved 5 minutes at 108.4°C. (5 lb. per sq. in.). After cooling the volume was made to 250 ml. with distilled water and the whole mixed thoroughly. An aliquot of the supernatant liquid was taken, diluted to bring its activity per mil in the neighbourhood of that of the standard solution, and filtered.

The latter solution was submitted to analysis. *The extraction technique* is the important feature around which most of the work described in the present paper revolves.

With the exception of Section 1 of the experimental data reported below, the paper relates experiments made in order to study this phase of the analysis by comparing the results obtained by using various modifications thereof.

Excepting the experiments related to enzymatic digestion, all the variants were modifications of the autoclaving phase itself. Either a reducing agent was added to the material prior to autoclaving or this reagent was added to the

50 ml. of water used for this operation; in some cases the water was replaced by culture medium with or without the addition of reducing agent.

(*b*) MICROBIOLOGICAL ASSAY TECHNIQUE. The organism used was *Lactobacillus leichmannii* A.T.C.C. 4797 and the basal medium is shown in Table I. Except for a slight modification (no tryptic digest) it is equivalent to that proposed by Skeggs *et al.* [8]. Besides, thioglycolic acid (50 mg. per liter) was added just prior to use.

The test culture was subcultured in the broth stock medium plus dried cod liver extract 1.5 g. per 100 ml. Cod liver extract gives the same growth as beef liver extract.

TABLE I. Composition of medium.

Component	Quantity for 1000 ml.	Component	Quantity for 1000 ml.
Potassium dihydrogen phosphate	0.5 g.	Calcium pantothenate	1.3 mg.
Potassium monohydrogen phosphate	0.5 g.	Niacin	1.3 mg.
Magnesium sulfate ($7H_2O$)	0.4 g.	Folic acid	0.6 mg.
Sodium acetate cryst.	12.0 g.	Cystine hydrochloride	100.0 mg.
Glucose	20.0 g.	Cysteine hydrochloride	150.0 mg.
Acid hydrolysed casein	10.0 g.	Tryptophane	130.0 mg.
Sodium chloride	10.0 mg.	Adenine	5.5 mg.
Ferrous sulphate	10.0 mg.	Guanine	5.5 mg.
Manganous sulphate ($4H_2O$)	10.0 mg.	Uracil	5.5 mg.
Pyridoxine	2.4 mg.	Xanthine	5.5 mg.
Pyridoxal	4.8 mg.	Biotin	6.5 μg.
Riboflavin	1.3 mg.	Tween 80	0.5 ml.
Thiamine hydrochloride	1.3 mg.	Water	to one liter

The inoculum was prepared from 6 ml. of a 24-hour culture of *L. leichmannii* in broth medium. It was centrifuged, the cells washed twice with sterile water or physiological saline and resuspended in 40 ml. sterile distilled water. The standard solution was made of 0.5 μg. per liter crystalline B_{12}. Assays of standard and unknown were carried out each in duplicate at four different levels: 0.25, 0.50, 0.75, 1.00 ml., and one blank was included in every series of standard solutions. To each tube was added: water to 1 ml. and 5 ml. basal medium. The tubes were sterilized by steaming 15 to 20 minutes. After cooling, one drop of the inoculum was added to each tube and the whole was incubated 24 hours at 37°C. Then 5 ml. water were added to each, and turbidities were determined with the Coleman Junior Spectrophotometer at 650 mμ. in 25-mm. diameter tubes. Blanks read 95-98 per cent transmission.

In calculating the value of the activity, suitable corrections are applied for moisture and fat content in the original material. It is emphasized that these values do not represent the vitamin B_{12} content of the samples examined but the "vitamin B_{12} activity" expressed in μg. of pure vitamin B_{12}. More specifically, it is the vitamin B_{12} activity for *L. leichmannii* A.T.C.C. 4797.

EXPERIMENTAL

The experimental data given below have been divided into four sections. Section 1 deals with the influence of heat and pH conditions on the B_{12} activity of cod liver residue. Section 2 relates our attempts to release and to protect the factors in the course of extraction with water prior to the analysis. The third section describes our experiments with sodium bisulphite. In the fourth section the results obtained by using bisulphite are compared to those obtained by using ascorbic acid.

Section 1. Effect of Alkali and Heat Treatment on the B_{12} Activity of Cod Liver Residue

Dry, defatted cod liver residue which had shown a vitamin B_{12} activity corresponding to 2.9 μg. B_{12} per g. was emulsified in 100 ml. of water. Aliquots of this emulsion, adjusted to various pH values, were kept at 60°, 80° and 100°C. respectively for 30 minutes.

Water solutions of known crystalline vitamin B_{12} content were treated similarly. The results of the comparative assays are given in Table II.

Table II shows that at 60°C. the losses are very comparable in both cases except at pH 12 for crystalline vitamin B_{12} where the loss is higher. At 80°C., and up to pH 11.15, losses are again comparable, but crystalline B_{12} is much less resistant beyond that pH. At 100°C. the loss is near total at all pH values for

Table II. The effect of alkali and heat on vitamin B_{12} solutions and cod liver residues.

Crystalline B_{12} 0.5 μg. per liter

Temp.	60°C.		80°C.		100°C.	
pH	Recovery	Loss	Recovery	Loss	Recovery	Loss
	μg./g.	%	μg./g.	%	μg./g.	%
11.00	0.392	22	0.336	33	—	—
11.15	0.392	22	0.336	33	0.085	83
11.3	0.392	22	0.300	40	0.030	94
11.6	0.392	22	0.180	64	0.015	97
12.0	0.280	44	0.064	87	0	100

Fat-free liver meal, B_{12} activity 2.9 μg. per g.

pH	Recovery	Loss	Recovery	Loss	Recovery	Loss
	μg./g.	%	μg./g.	%	μg./g.	%
11.00	2.4	18	2.1	28	0.7	75
11.15	2.3	20	2.1	28	0.6	80
11.3	2.3	20	2.2	23	0.6	80
11.6	2.3	20	2.1	28	0.6	80
12.0	2.2	23	2.0	32	0.3	90

vitamin B_{12}, while under the most drastic conditions there is still 10 per cent activity left in the liver material. This 10 per cent activity represents alkali-stable factors present in the liver material. If the results in Table II are recalculated by taking into account the presence in the liver material of 10 per cent alkali-stable activity, one obtains the losses in alkali-labile activity (Table III). When the new figures are compared to the values obtained for crystalline vitamin B_{12}, the following picture emerges: the alkali-labile activity of the liver between 60° and 80°C. is more stable than crystalline vitamin B_{12} under the same pH conditions. However, for both materials, destruction occurs readily at 100°C. over the whole range and is complete at pH 12.

TABLE III. The percentage loss in activity of the alkali-labile factors in liver residue (L) as compared to crystalline vitamin B_{12} under the same conditions of temperature (30 min. heating) and pH.

	Percentage loss									
	pH 11		pH 11.15		pH 11.3		pH 11.6		pH 12	
Temp. °C.	L	B_{12}	L	B_{12}	L	B_{12}	L	B_{12}	L	B_{12}
60	21	22	24	22	23	22	22	22	25	44
80	31	32.8	31	32.8	25	40	31	64	36	87
100	83	..	88.5	83	88.5	94	90	97	100	100

The effect of exposure to lower pH values was studied on a sample of liver residue obtained from livers that had been kept in cold storage for some months (−20°C.). The material was divided into three lots. One was adjusted to pH 5.0, another to pH 6.7 and the third to pH 9.0 From each lot a series of 5-g. samples was weighed. The results of assays made on the samples after they had been subjected to steaming for various lengths of time are recorded in Table IV. The figures express in μg. the amount of vitamin B_{12} equivalent to the activity of the samples.

TABLE IV. The effect of pH on the vitamin potency of steamed cod liver residue. Potency expressed as equivalent of μg. B_{12} per 5 g. residue.

Steaming time (minutes)	0	10	20	30	40	50	60
pH 9	0.88	0.94	0.90	0.90	0.86	0.80	0.84
pH 6.7	0.92	0.84	0.88	0.86	0.90	0.86	0.82
pH 5	0.84	0.88	0.96	0.92	0.92	0.90	0.90

It can be seen that there is no highly significant difference, the average of all the determinations being 0.88 μg. As this does not differ significantly from the value obtained on the original material without treatment (0.84 μg.), one can only conclude that steaming at a pH within the range 5-9 does not result in a decrease in vitamin B_{12} activity over a period of one hour.

Another experiment was then carried out in order to determine eventual losses of activity at pH 8 and pH 9 respectively with prolonged heating at 60°C., conditions likely to be met in practice. This was effected with 5-g. samples of a liver residue prepared from fresh livers. The technique was identical with that used in the preceding experiment.

Table V shows the results of this experiment. This table is interesting in that it shows a decline in activity after one hour of heating in both cases but more definitely at pH 9; yet this first decline was only apparent since the value rose steadily in both cases on continued heating. Both samples were allowed to

TABLE V. Effect of prolonged heating (60°C.) at pH 8 and pH 9, respectively, on liver residue. Initial activity: 2.62 μg. per 5 g.

pH	1 hour	3 hours	5 hours
8	2.02	2.16	2.34
9	1.43	2.00	2.16

cool after five hours of heating and left at room temperature over night. Five-gram samples were then weighed from each lot and analysed. They showed at pH 8 and pH 9, 2.42 μg. and 2.50 μg. respectively, values close to the initial one.

SECTION 2. ENZYMATIC LIBERATION OF THE GROWTH FACTORS

The effect of enzymes, pepsin and papain at pH 5, and the pancreatin at pH 7.2 were studied.

Samples of liver protein residue were sterilized by autoclaving for five minutes at 108.4°C. (5 lb. per sq. in.). The enzymes, suspended in sterile water, were added aseptically to the cooled samples which were then covered with a thin layer of toluene and incubated at 37°C. for 24 hours.

The results showed no effect for pepsin or papain, and an actual decrease in activity from the equivalent of 1.78 μg. B_{12} to 0.95 μg. in the presence of pancreatin.

EFFECT OF ANTIOXIDANTS. Three samples of a homogenized cod liver residue were admixed with 0.1 per cent of their weight of ascorbic acid, sodium thiosulphate and sodium bisulphite, respectively. Each was then immediately assayed according to the method described above. Table VI shows the effect of three antioxidants. Activities are expressed in μg. vitamin B_{12} for 1 g. dry, defatted material.

TABLE VI. Effect of antioxidants: ascorbic acid, sodium thiosulphate, sodium bisulphite. Values expressed in μg. vitamin B_{12} per g.

Sample	No. addition	Ascorbic acid 0.1%	$Na_2S_2O_3$ 0.1%	$NaHSO_3$ 0.1%
A	.38	.31	.46	1.17
B	1.76	—	—	3.02
C	.32	—	—	.39

Treatment with ascorbic acid is detrimental; a certain increase was observed with sodium thiosulphate but a large increase resulted from the treatment with bisulphite.

SECTION 3. THE ACTION OF SODIUM BISULPHITE

In order to determine whether the influence of bisulphite was not due to some growth-promoting action independent of the factors studied, standard curves were duplicated with and without additions of sulphite to the basal medium for a series of assays. The standards showed in all cases the same growth level in all pairs of tubes, and the curves were identical.

In Table VII are shown some results obtained by submitting a standard solution of vitamin B_{12} 0.5 μg. per liter to various heat treatments with and without $NaHSO_3$. No significant change was observed except on autoclaving, when, in the absence of buffer, complete destruction was obtained. The protective

TABLE VII. Recovery of vitamin B_{12} from a solution of 0.5 μg. per litre after various heat treatments with or without added sodium bisulphite.

	Unheated		Steamed 10 min.		Autoclaved 5 min./5 p.s.i.		
Sodium bisulphite, mg. per litre	0.0	0.5	0.0	0.5	0.0	0.5	0.5
Vitamin B_{12} μg. per litre	0.50	0.50	0.45	0.48	0.48	0.00	+ acetate buffer 0.38

action of the buffer explains why when samples of natural materials are submitted to autoclaving in the presence of $NaHSO_3$ during the extraction phase of the assay procedures, no drastic decrease in activity occurs, presumably, because of the buffering properties of the protein substrate.

An experiment was carried out with bisulphite on haddock liver residue. Table VIII shows the results obtained from two different samples using different levels of bisulphite.

TABLE VIII. Effect of sodium bisulphite on the release of vitamin in the extraction of liver residue.

$NaHSO_3$	0.00%	0.01%	0.02%	0.04%	0.05%	0.08%	0.10%	0.15%
Sample A	0.71	—	—	—	0.95	—	1.63	—
Sample B	0.28	0.29	0.31	0.33	—	0.37	0.34	0.30

The following experiment was carried out in order to determine optimum conditions for the extraction of samples submitted to analysis. A certain amount of homogenized cod liver residue was divided into two lots. To one was added bisulphite (0.08 per cent by weight). Then, from each lot, 5-g. samples were taken and submitted to extraction under the conditions described in Table IX, which shows the results obtained for bisulphite treated and untreated samples, at various pH values, either after steaming or autoclaving.

TABLE IX. Effect of pH, bisulphite addition, steaming and autoclaving in the extraction of cod liver residue. Activity in μg./g.

pH	5.5		6.7		8.5	
$NaHSO_3$	0%	0.08%	0%	0.08%	0%	0.08%
Steamed 15 min.	1.03	1.32	0.98	1.32	1.26	1.74
Autoclaved 5 min./5 p.s.i.	1.03	1.94	.96	1.32	1.32	1.62

The table shows that the highest results are obtained under the conditions applied in the extraction phase of the assay, namely, pH 5.5, autoclaved 5 minutes at 5 pounds pressure (see p. 130), provided that bisulphite is present.

Table X shows the results of comparative assays effected with and without the addition of 0.01 per cent bisulphite to samples of materials of widely different origin. The results are expressed in μg. of vitamin B_{12} per gram dry, defatted material.

It appears from the results described in Table X that the effect of bisulphite is not merely confined to cod liver residues, but is of general application.

The total increase due to bisulphite is in some cases much less pronounced. It was found, for instance, that bisulphite had very little effect when used with air-dried material. However, when it is added prior to drying, the dry material obtained still leads to the same increased values even after several months of storage.

TABLE X. Comparative assays with and without addition of bisulphite for natural materials of various origin. Activity in μg. B_{12} per g. dry, fat-free material.

	Without	With		Without	With
Cod liver residue	0.90	2.90	Chicken faeces	0.32	.35
Pork liver	0.61	0.79	Cod stomach	0.91	1.21
Hog faeces	2.00	5.28	Content of cod stomach	0.37	0.51
Beef liver	3.97	5.26	Cod intestine, upper part	0.34	0.35
Cow faeces	.92	1.80	Cod intestine lower part	0.34	0.36
Horse faeces	.13	—			

SECTION 4. COMPARISON BETWEEN THE EFFECT OF SODIUM BISULPHITE AND THE EFFECT OF ASCORBIC ACID

Homogenized cod liver residue was divided into two lots. One lot was untreated while the other was admixed with 0.08 per cent $NaHSO_3$. From the $NaHSO_3$ treated material two 1-gram samples were weighed and from the untreated residue, four.

One gram sample of $NaHSO_3$-free material was suspended in 50 ml. water (see above "Preparation of Samples").

Another was suspended in 50 ml. of 0.02 per cent aqueous solution of ascorbic acid.

A third was suspended in 50 ml. of the assay medium as described in Table I (no addition of thioglycolic acid).

The fourth was suspended in 50 ml. of assay medium containing 0.02 per cent of ascorbic acid.

One of the $NaHSO_3$-treated samples was suspended in 50 ml. of water; the other was suspended in 50 ml. medium (Table I; no thioglycolic acid added).

The six mixtures were then autoclaved simultaneously for 10 minutes at 121°C. (15 lb. per sq. in.). At this point, the usual analytical technique was followed except that 0.02 per cent thioglycolic acid (instead of the usual 50 ml. /liter) was added to the assay medium.

The change in autoclaving temperature and in the thioglycolic acid content of the assay medium were made in order to bring the conditions closer to those suggested by the work of Hendlin and Soars [2].

Of the resulting extracts, several dilutions were made so that at least one dilution would be found of the same order of activity as the standard solution. The results obtained with such dilutions were those retained and are given in Table XI. This table gives, in addition, the results obtained by applying the whole procedure to three other samples of cod liver residue.

It is seen that medium as such has an advantage over water, that ascorbic acid in water is very detrimental, whereas in the presence of medium a large increase is obtained. However, $NaHSO_3$ and water gave in general an even

TABLE XI. Comparison between the effect of ascorbic acid and sodium bisulphite with and without medium in the course of autoclaving. B_{12} activity values expressed as μg. B_{12} per g. of original residue.

Conditions applied	Sample No.			
	I	II	III	IV
Water alone	0.275	0.220	0.290	0.370
Water + ascorbic acid	0.200	0.135	0.135	0.109
Medium alone	0.264	0.378	0.355	0.443
Medium + 0.02% ascorbic acid	0.258	0.516	0.414	0.500
$NaHSO_3$ and water	0.340	0.570	0.492	0.565
$NaHSO_3$ and medium	0.400	0.510	0.493	0.576

larger increase whereas the presence of medium with the same reagent does not have a beneficial effect.

The results obtained with the first sample are somewhat out of line with those obtained in the three following series; they are less significant, however, because the activities of the dilutions made were not close enough to the standard solution activity, as it was our first experiment of this kind.

DISCUSSION

The experiments described in part I of the experimental section (Tables I, II, III and V) show clearly the advantage of the new rendering process over the alkali-digestion process. While the latter ($pH > 10$, 100°) is very destructive, the range of conditions prevailing in the former ($pH \leqslant 8.5$, 60°) does not appear detrimental. Yet, under the most drastic conditions, about 10 per cent of the total initial activity remains unaffected, indicating the presence of alkali-stable factors.

Table V shows a very interesting fact: under sustained treatment at pH 8 or pH 9 at 60°C., the activity declined to about 50 per cent of its initial value, then rose steadily to resume the latter. The "losses" indicated by the analysis at intermediate stages were thus only apparent, and we had good reasons to believe that the procedure could not discriminate between true losses and changes which did not invbolve actual destruction; in fact, we could expect the state corresponding to such changes to result from other treatments or even to exist to some extent in any natural material and therefore we could suspect any value obtained through this method.

Anomalously low results may arise from any of the following causes:

1. A variable binding of the factors to the protein substrate.
2. A change of the factors into forms inactive on *L. leichmannii*.
3. A change into forms subsequently destroyed under the analytical conditions.

In favour of hypothesis 1 are the following facts:

(*a*) The sample is not analysed as such; instead, an aqueous extract of the material, made by autoclaving, is submitted to the microbiological assay. Incomplete extraction thus leads to low results.

(*b*) Types of proteins are known to bind vitamin B_{12} [4, 5, 7].

(*c*) Difficulties in the extraction of vitamin B_{12} active factors from natural substrate are acknowledged by all workers in the field.

(*d*) In some cases, enzymatic proteolysis has led to distinctly higher values [6].

In favour of hypothesis 2 is the fact that some vitamin B_{12}-protein combinations, inactive on *L. leichmannii,* are water soluble and therefore would be taken up by the extract. It is likely, however, that such combinations would be split under the treatment the material undergoes during the analysis (autoclaving) [7].

Hypothesis 3 is supported by the fact that vitamin B_{12a} and vitamin B_{12b} are sensitive to heat treatment. As shown by Hendlin and Soars [2], deactivation occurs on autoclaving in the presence of assay medium unless a reducing agent (thioglycolic acid or ascorbic acid) is added to the medium.

We have no proof that our material contains B_{12}, B_{12a} and/or B_{12b}, but as this is very likely we shall assume provisionally that it may contain these factors and see how the results can be explained by the help of this hypothesis.

The anomalies observed in our experiments cannot be ascribed to possible deactivation of B_{12a} and/or B_{12b} during the *sterilization* process of the assay tubes, because thioglycolic acid was present in our medium. Incidentally, this addition, suggested in an article by Hoffman *et al.* [3] did not bring any change in our results.

Deactivation of B_{12a} and/or B_{12b} can also occur during the *extraction phase* (autoclaving); this would explain the inefficacy of thioglycolic acid in the next step (sterilization).

Very early we suspected that the extraction phase of the analysis was the source of the trouble. Enzymatic liberation having failed to lead to any improvement (Table V), we tried the addition of several antioxidants or reducing agents. As seen in Table VI, all failed except sodium bisulphite which brought about a large increase in the results (Tables VIII, X, XI). At the time these experiments were conducted, the work of Hendlin and Soars [1] had not been published. The failure of ascorbic acid (and perhaps other agents) can now be explained, in the light of their findings, by the absence of medium, since B_{12a} and B_{12b} would be destroyed under those conditions by ascorbic acid while B_{12} would remain unaffected. These conclusions fit very well the data shown in Table XI, on the hypothesis that our material contains B_{12}, B_{12a} and/or B_{12b}.

To ascribe the large increase resulting from autoclaving in the presence of medium containing 0.02 per cent ascorbic acid to the protection of B_{12a} and/or B_{12b} would then seem a natural conclusion. The effect of sodium bisulphite could also be, at least partly, due to a similar property. The remarkable fact is that sodium bisulphite does not require the presence of medium to be effective

and the results are higher than those obtained with ascorbic acid plus medium as shown by Table XI. These results seem to indicate that some additional effect is involved and also that the mechanism may be different.

Thus, while hypothesis 3 above explains in part the action of sodium bisulphite, there remains the problem of accounting for the higher results obtained with this reagent. We suggest a release-promoting action of the factors from the substrate (in relation to hypothesis 1) or an additional protective effect not offered by ascorbic acid plus medium, possibly on a factor different from B_{12a} and/or B_{12b}.

CONCLUSIONS

While there is no final explanation for the action of $NaHSO_3$, the latter appears to be, along with thioglycolic acid and sodium cyanide [1] a valuable analytical tool. It would certainly prove important if it were demonstrated that the results obtained through its use more closely parallel those obtained for the Animal Protein Factor as determined by animal assay.

It seems indicated to conduct the extraction phase of microbiological assays (autoclaving) in the presence of ascorbic acid plus medium or in the presence of sodium bisulphite.

Comparative assays made on the same natural material and involving autoclaving with water, water plus ascorbic acid, medium plus ascorbic acid, water plus $NaHSO_3$ and water plus caustic alkali may prove valuable as the basis of a method for estimating the different B_{12}-active constituents.

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The First Record of the Smooth Lump-Sucker, Cyclopterichthys ventricosus (Pallas), in British Columbia Waters

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A specimen of the above species of fish was brought by Mr. Grant Robertson to the Department of Zoology, University of British Columbia for identification and proved to be a new record for British Columbia waters. Mr. Robertson kindly permitted the writers to prepare the description and our thanks are extended to him for this privilege. Subsequently a second individual was forwarded by Fisheries Inspector J. A. Summers, to whom we are greatly indebted.

The account has been prepared in the form used in the bulletin on the marine fishes of British Columbia (Clemens and Wilby, 1946). The illustration is the work of Mr. F. L. Beebe.

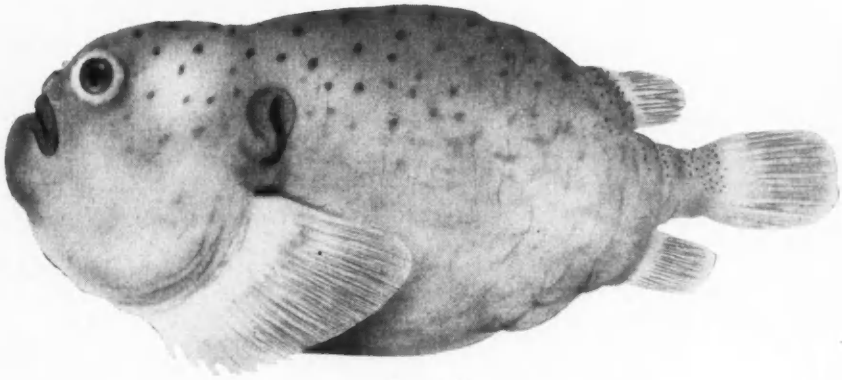

Figure 1

Smooth Lump-sucker—*Cyclopterichthys ventricosus* (Pallas) 1769 (Figure 1)

Body short, deep, stout anteriorly, compressed posteriorly. Head globular, bluntly rounded; mouth terminal, wide, oblique; teeth in two rows anteriorly, outer small continuous, inner larger, irregular; snout broad, profile curved; nostrils paired, anterior tubular; eyes small; interorbital space flat, broad. Opercular opening very small, entirely above base of pectoral fin. Skin smooth, thick, loose. Fins: dorsal (1), 8 or 9, far back on body; anal, 7 to 9; pelvics thoracic, modified into moderate adhesive disc, with thickened margin; caudal rounded. Lateral line: absent. Scales: absent. Cirri: absent. Colour: brownish gray, spotted with black on dorsal surface; muddy gray on ventral surface.

Length to 5½ inches.

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Distinguished by the short body and globular head, the absence of tubercles, the smooth loose skin, the single, rayed dorsal fin.

The smooth lump-sucker was first taken in British Columbia waters on August 1st, 1950, in Dean Channel in a salmon seine. A second individual was discovered in early September, 1951, in a salmon purse seine on the west coast of Moresby Island by Fisheries Inspector J. A. Summers. This specimen is in the Department of Zoology, University of British Columbia. These are the only records for British Columbia and apparently the only records south of the Bering Sea.

Range: Northern British Columbia to Northwestern Alaska.

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The Agonid Fish Pallasina barbata aix (Starks), from British Columbia

By W. E. Barraclough
Pacific Biological Station, Nanaimo, B.C.
(Received for publication September 19, 1951)

Five specimens of the agonid fish *Pallasina barbata aix* (Starks) have been recovered from three localities in British Columbia waters. The first was caught in a small beach seine, July 24, 1947, in Whitesand Cove, on Flores Island off the west coast of Vancouver Island, by Mr. K. S. Ketchen of the Pacific Biological Station. A second specimen was recovered from a pan of frozen herring bait by Mr. A. L. Murray, fishery officer of District No. 1, Vancouver, B.C., on April 10, 1948, and donated to the University of British Columbia. The precise location of capture of this specimen is uncertain but it was known to have been caught by a herring seiner fishing in shallow water near Alert Bay on the east coast of Vancouver Island. A third specimen was caught by Captain George Gerbrandt of the shrimp trawler "Guillotte A," while trawling at a depth of 30 fathoms on the west side of Turnour Island in Johnstone Straits. Mr. R. M. Wilson of the Pacific Biological Station recovered this specimen for identification on April 10, 1948. Two specimens were collected by the author in a beach seine on the east side of Vargas Island off the west coast of Vancouver Island on August 31, 1951. The size and other characteristics of the five specimens are given in Table I, and Figure 1 is an illustration of the species drawn by Mr. D. Denbigh.

Table I. Length, fin rays, and number of unpaired plates on a median line of the breast in front of the pelvic fins, for the five specimens.

Origin of specimen	Whitesand Cove	Alert Bay	Turnour Island	Vargas Island	
Standard length, mm.	68	83	91	65	63
Dorsal fin rays	VII, 8	VII, 8	VI, 7	VI, 8	VII, 7
Anal fin rays	12	12	11	11	11
Pectoral fin rays	11, 12	12, 12	12, 12	11, 12	11, 11
Prepelvic plates	2	2	2	2	2

This species was first described by Steindachner (1876, p. 188, pl. 5) as a new species and named *Siphagonus barbatus*. It was collected from the Arctic region just north of the Bering Strait. Steindachner also recorded the species from Hakodai and Nagasaki, Japan. Bean (1882, p. 248; 1883, p. 25) records S. *barbatus* from Port Mulgrave, Yakutat Bay, Unalaska and Port Clarence, Alaska. Jordan and Gilbert (1882, p. 725) list the species from the coast of Alaska and Japan. Jordan (1885, p. 901) places the species among the Arctic

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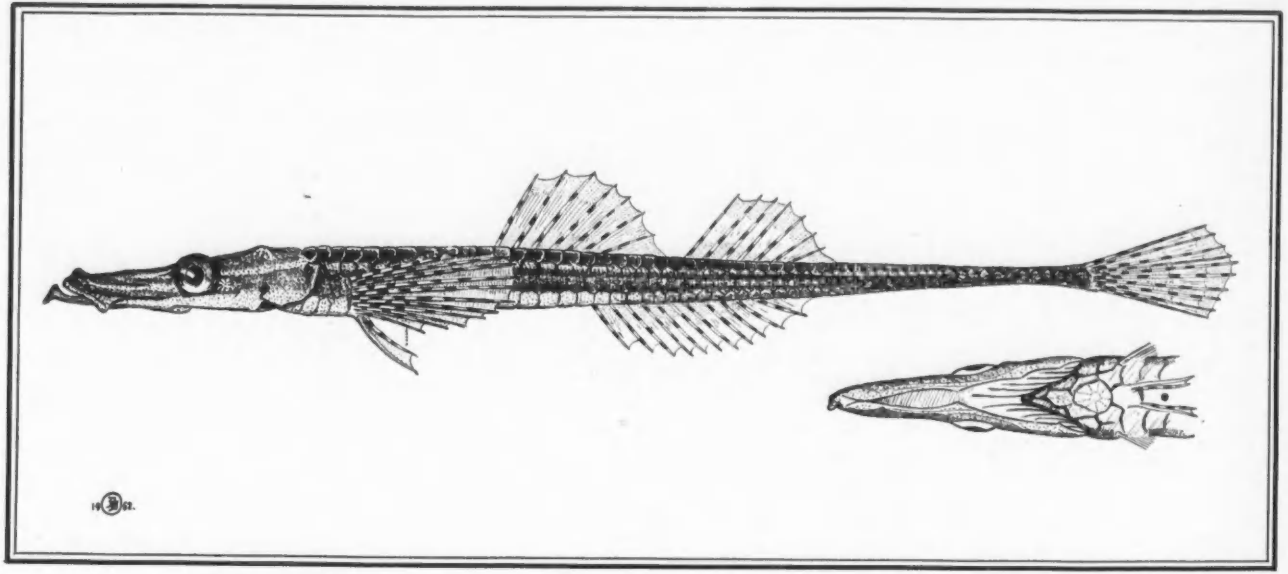

FIGURE 1. General appearance of *Pallasina barbata aix* (Starks), drawn by Mr. D. Denbigh.

fauna. Turner (1886, p. 94) lists the species from Iliuliuk, Unalaska. Gilbert (1895, p. 435) obtained *S. barbatus* in the ichthyological collections of the steamer "Albatross" at seven stations in Bristol Bay, at depths from 4.5 to 70 fathoms. He also collected the species during the summer of 1891 with a seine at Port Angeles, Washington. The species is tabulated by Schmidt (1903, p. 58) from the Sea of Japan, the Sea of Okhotsk, and the Bering Sea.

Most of the species formerly included in the genus *Siphagonus* are now placed in *Pallasina* by Cramer (in Jordan and Starks, 1895, p. 815), because *Siphagonus* was based on *Agonus segalienis*, which seems to be a true *Brachyopsis*. *Pallasina* was named for Petrus Simon Pallas, naturalist and explorer, the author of *Zoographia Rosso-Asiatica*, 1811. It is distinguished from *Brachyopsis* by the long slender body, syngnathous snout, and by the presence of a long barbel on the chin.

Jordan and Snyder (1901, p. 368) include *P. barbata* in a list of fishes collected in Japan by Keinosuke Otaki, and the United States steamer "Albatross". Additional specimens of *P. barbata* are described by Jordan and Starks (1904, p. 588) from Aomori, Nemuro, and Mororan, Japan. The authors state that this species is the most abundant of the Agonidae of Japan, occurring in eel grass in shallow bays. Later, Snyder (1912, p. 436) records *P. barbata* as being collected by the United States Bureau of Fisheries steamer "Albatross" from Mori, Mororan, and Hakodate, Japan. Everman and Goldsborough (1907, p. 328) record 12 specimens seined in Funter Bay; 1 seined in Kilisut Harbor, 2 seined in Cleveland Passage; all collected by the "Albatross" in 1903 in Alaska. They also record specimens of *P. aix* that were collected by Scofield (1899, p. 504) from Chignik Bay, Alaska.

Pallasina aix was described as a new species by Starks (1896, p. 558, pl. 75), on the basis of its shorter barbel and 2 instead of 3 prepelvic plates. This species was reported to have been taken with a seine in great abundance in Puget Sound near Port Ludlow, Washington. Starks' characters were incorporated into a key by Jordan and Evermann (1898, p. 2049). Jordan and Gilbert (1899, p. 471) record two specimens of *Pallasina barbata* from Shana Bay, Iturup Island, Japan, and one from Tareinsky Bay, Kamchatka, and mentioned that Dr. Stejneger found the species at Petropaulski, Kamchatka. These specimens typically had 3 prepelvic plates as described for *barbata*, not 2 as in *aix*. Scofield (1899, p. 504) records six specimens of *P. barbata* from Port Clarence, Alaska, and finds that the specimens have much longer barbels and slightly longer pectoral fins than specimens from Bristol Bay. Scofield also lists two specimens as *P. aix*, taken at Chignik Bay, Alaska; one had 3 prepelvic plates, the other had 2.

Starks (1911, p. 194) still considered *P. aix* to be specifically distinct from *P. barbata*, on the basis that the length of the mandibular barbel was less variable in specimens from Puget Sound than in Alaskan specimens. He states in addition that either 2 or 3 prepelvic plates may occur among both species: but for *aix* 2 is the usual number and 3 the exception, while for *barbata* 2 is the exception.

Gilbert and Burke (1912, p. 65) suggest that *P. aix* be reduced to subspecific rank. Their suggestion resulted from a comparison of many specimens previously collected from Kamchatka, Alaska and Puget Sound. They found that the number of dorsal rays, anal rays, dorsal spines, pectoral rays and the number of unpaired prepelvic median plates show a marked overlapping and intergradation between different localities. Specimens from each locality seem to take on slightly distinctive combinations of the above characteristics.

It would appear that probably the best subspecific characteristic for distinguishing *Pallasina barbata aix* from *Pallasina barbata barbata* is the presence of 2 unpaired plates on the median line of the breast in front of the pelvic fins. The length of the barbel on the chin is evidently not a reliable characteristic for distinguishing the two subspecies, although a shorter mandibular barbel seems to occur more uniformly in specimens of *P. b. aix*. The suggestion of Gilbert and Burke is therefore used in recording this new record of five specimens of *Pallasina barbata aix* from British Columbia.

Most sincere thanks are due Dr. W. A. Clemens, Head of the Department of Zoology at the University of British Columbia for lending the specimen from the collection of the museum at the University for examination. The author wishes to thank Mr. K. S. Ketchen and Mr. R. M. Wilson for kindly presenting the two specimens to the author for examination. These two specimens are now in the collection of fishes in the Museum of the Pacific Biological Station, Nanaimo, B. C. The helpful suggestions of Dr. Carl L. Hubbs are gratefully acknowledged, and also his examination of the specimens with the author.

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The Importance of Flavobacterium in Fish Spoilage

By C. H. Castell and E. G. Mapplebeck
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(Received for publication July 20, 1951)

ABSTRACT

Two hundred and forty-five cultures of *Flavobacterium* were isolated from fish; 16 per cent reduced trimethylamine oxide; 25 per cent reduced sodium nitrate; 50 per cent were proteolytic; 70 per cent grew at 2°C. None fermented dextrose or hydrolysed fat.

Many of the cultures produced a yellow discoloration but no offensive spoilage odours on fish muscle. Other strongly proteolytic cultures produced putrid odours at 5 to 8 days on fish stored at 3°C.

Under commercial conditions they are not as important in the development of spoilage as the more active achromogenic rods which rapidly outgrow the *Flavobacterium* on stored fish.

INTRODUCTION AND METHODS

Bacteria of the genus *Flavobacterium* are among the organisms most frequently encountered on fresh fish. They are also present in soil, fresh water, sea water and ice. There is reason to believe that their abundance in the environment of the fish accounts for their presence in the gills and the slime of the freshly caught fish.

Their presence on fish has been observed by almost all workers who have

Table I. Percentage of *Flavobacterium* among colonies isolated from fish by various workers.

Author	Source	*Flavobacterium*
		%
Fellers (1926)	salmon	20.6
Reed and Spence (1929)	haddock faeces	5.6
Reed and Spence (1929)	haddock slime	8
Stewart (1932)	haddock slime	11
Stewart (1932)	haddock faeces	2
Stewart (1934)	haddock slime—7 days on ice	20
Shewan (1949)	haddock slime—fresh	6
	haddock slime—24 days on ice	0
Bedford (1933)		33
Thjotta and Somme (1938)	cod	25
Snow and Beard (1939)	salmon	4.9
Dyer (1947)	cod slime and faeces	3
Wood (1940)	freshly caught fish	50
Wood (1940)	fish in retail store	3
Anderson (1947)	freshly cut fillets	33.4
	spoiled fillets	3
Aschehoug and Vesterhus (1943)	fresh herring slime	17.7
Aschehoug and Vesterhus (1943)	fresh herring gills	13.7
Aschehoug and Vesterhus (1943)	fresh herring faeces	0
Aschehoug and Vesterhus (1947)	herring muscle—6 to 11 days at 1° to 8°C.	7
Bedford (1933)		30
Pivnick (1948)	fresh cod fillets	43.1

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done systematic studies on the microflora of marine products (Fellers, 1926; Harrison, 1929; Sanborn, 1930; Gibbons, 1934; Schonberg, 1938; Kimata, 1942; etc.). Table I gives a summary of the observations of several workers regarding the prevalence of the Flavobacteria on various types of fish.

As a group, members of the genus *Flavobacterium* are characterized by rather limited activities. They all produce yellow or orange pigments. Along with the other members of the Achromobacteriaceae, they have very feeble powers of attacking carbohydrates. Snow and Beard (1939) found that none of the cultures they isolated was able to hydrolyse fats. Of the few species tested by Baird (1943), none was able to reduce trimethylamine oxide. Of the 27 species listed in Bergey's manual (1948), 20 are able to liquefy gelatin and five produce hydrogen sulphide.

Many members of the genus *Flavobacterium*, especially those of marine origin, are able to grow at low temperatures (0° to 5°C.). Hess (1934) observed that *Fl. deciduosum*, which he isolated from fish, would grow and produce its yellow pigment at –6.5°C. and would slowly decompose proteins at –3°C.

Wood (1940) pointed out that there is a succession in the prevailing genera in fish as it changes from a fresh to a spoiled condition. He noted that species of the *Flavobacterium* and the *Micrococcus*, which are most abundant in fresh fish are gradually superseded by *Achromobacter* and *Pseudomonas* as spoilage develops.

There is little definite information regarding the part played by species of the *Flavobacterium* in the spoilage of fish. Schonberg (1938) considered them to be important, because of their proteolytic activities and their ability to grow at low temperatures. Elliott (1947) suggested that members of the *Flavobacterium* and the *Micrococcus* may be responsible for the initial "flat", "sweet" and "stale" odours which develop in fish before it becomes putrid through the activities of the colourless Gram-negative rods. However, by inoculating sterile fish with pure cultures of fish-spoiling bacteria, Castell and Anderson (1948) noted that a similar succession of odours from "stale" and "sweet" to "putrid" or "faecal" could result from the activities of single cultures of *Proteus*, *Pseudomonas*, *Serratia* or *Achromobacter*. They also observed that in similar experiments with some pure cultures of *Flavobacterium* on fish at 3°C., the spoilage odours were slower in developing and much less offensive than those produced by *Proteus*, *Pseudomonas* or *Achromobacter*.

These observations may be summed up briefly by stating that, although it has been definitely established that species of *Flavobacterium* are among the principal species found on fish, especially freshly caught fish, there is little conclusive evidence of the part they play in the normal spoilage of fish. The work recorded in the present paper has been done with the object of throwing a little further light on this subject.

Isolation of Cultures

Agar plates were poured from 56 samples of the skin and muscle of fresh

and frozen cod and haddock. From each set of plates, representative yellow and orange colonies were picked off and, after initial microscopical examination, the cocci were discarded. From the remaining rod-types, 245 cultures were obtained having the general characteristics of *Flavobacterium*. Only six were definitely Gram-positive, the remainder being either Gram-negative or Gram-variable. The majority of the cultures were composed of rod-shaped cells, but there were also many that showed extreme pleomorphism, and some that were composed of coccoid cells, difficult to distinguish from ordinary Gram-negative cocci.

An attempt was made to identify the species according to the classification given in Bergey's manual (1948). Although one or two of the named species were undoubtedly present, so many of the cultures could not be fitted into this recognized classification that the attempt was given up. However, on a basis of motility, morphology, pigmentation, ability to reduce trimethylamine oxide and nitrate, and to decompose proteins, and on their reaction in milk, there appeared to be at least 22 distinctly different strains or species. Of these, 12 were a diversified group of cultures of various shades of yellow. The remainder were orange or buff-coloured. Because these orange-pigmented cultures consisted of organisms that were apparently more closely related to each other, they were treated as a separate group.

Determining the Spoilage Potential

Two different methods were used to estimate the capacity of these organisms for spoiling fish. First, tests were made to determine their ability to hydrolyse fat, to reduce trimethylamine oxide, to decompose proteins (gelatin and casein), to ferment sugars and to grow at temperatures close to freezing. Theoretically, such information should give an approximate idea of their action on fish.

Secondly, in a more direct method, sterile cod muscle was inoculated with pure cultures of these organisms and incubated at 3° and at 25°C. Similar sets containing 0, 5, 8 and 12 per cent sodium chloride were incubated at 25°C. These were all examined daily for the production of off-odours and any physical change that might occur in the fish flesh.

Table II. The percentage of 132 yellow cultures and 113 orange cultures of *Flavobacterium*, isolated from fish, yielding positive results in tests likely to indicate their ability to spoil fish.

	Yellow	Orange
Reduce trimethylamine oxide	36	4
Reduce sodium nitrate	60	1
Ferment dextrose	0	0
Hydrolyse fat	0	0
Decompose protein	40	84
Growth at 2° to 3°C.	78	92
Growth in 5 per cent nutrient brine	40	8
Growth in 8 per cent nutrient brine	0	0

EXPERIMENTAL RESULTS

Table II summarizes the results of the tests using various culture media as the substrates. None of the organisms tested fermented either lactose, sucrose or dextrose. Many cultures in these sugar broths ended up with a strong alkaline reaction. None hydrolysed either butter fat or cod liver oil. Of the orange cultures, only a very few slowly reduced trimethylamine oxide or sodium nitrate. Almost half of the yellow cultures reduced nitrate and about one-quarter reduced the oxide. As a group, these organisms were much more active in their ability to decompose proteins (gelatin and casein). Almost all of the organisms grew slowly at 2° to 3°C. and produced their typical pigmentation.

The ability of the organisms to grow in broth containing various concentrations of sodium chloride was to some extent determined by the size of the inoculation. With a very light inoculation and up to twelve days incubation at 25°C., the results were as follows: 1, 2 and 3 per cent salt had little effect, or in some cases appeared to enhance growth; at 5 per cent salt about 40 per cent of the yellow cultures, but only 8 per cent of the orange cultures showed growth; with 8 per cent salt, no growth occurred with any of the cultures. These are definitely not very salt-tolerant organisms, and in this characteristic they differ from many of the yellow pigmented marine micrococci.

Action on Cod Muscle

Pieces of sterile fresh cod muscle, and also similar fish muscle that had been autoclaved, were inoculated by dipping them into suspensions of pure cultures. For comparison, similar fish samples were inoculated with suspensions of *Pseudomonas*. These were held in sterile glass containers, incubated at both 3° and 25°C. and examined periodically for spoilage odours.

At 25°C., in each case where the fish was inoculated with *Pseudomonas*, there was a pronounced off-odour within 24 hours and at 48 to 72 hours, the fish were quite putrid. The majority of the orange cultures of *Flavobacterium*, expecially those which were strongly proteolytic, had a pronounced off-odour at 48 to 72 hours, but not at 24 hours; at four to five days, most of these were extremely offensive. In some instances, the fish developed an orange colour at the surface, but the spoilage odours always preceded this discoloration.

The yellow cultures of *Flavobacterium* produced off-odours more slowly, and the odours themselves were much less offensive. At four to eight days many had developed a very characteristic glue-like odour. Others developed odours which were likened, by those judging them, to the odours of sea weeds, formaldehyde, almonds, etc. After an incubation period of two to three weeks, some developed the fishy odour of trimethylamine.

At 3°C. the fish inoculated with *Pseudomonas* became quite offensive at 48 to 72 hours. The orange cultures of *Flavobacterium* began to develop disagreeable odours at five to eight days and many became quite putrid by ten to eleven days. Fish inoculated with the yellow cultures developed a yellow discoloration between ten and fifteen days, but had no perceptible spoilage odours. At the end

of a month, at which time the experiment ended, many of the fish samples had an abundant growth of yellow organisms over the surface, but still had developed little or no spoilage odour.

With the orange cultures, wherever the concentration of salt permitted growth, the fish developed typical offensive spoilage odours. But this was not so for many of the yellow cultures. Some of these which produced mild spoilage odours with 0 to 5 per cent salt, grew on fish containing 8 per cent salt, to the point where the surface became yellow, without producing any noticeable odours.

Similar tests using *Pseudomonas* cultures, isolated from fish, produced very offensive odours at two, three and eight days in fish containing 0, 5 and 8 per cent salt. Other tests with *Aer. aerogenes, E. coli* and *Aer. cloaceae* showed rapid growth and offensive odours with fish containing 5 per cent salt but greatly retarded growth and relatively little odour with 8 per cent salt (Table III).

TABLE III. Production of spoilage odours from cod muscle containing 0, 5 and 8 per cent salt inoculated with pure cultures and held at 25°C.

Organisms	Days to produce offensive odours		
	0% NaCl	5% NaCl	8% NaCl
Yellow *Flavobacterium*	4–8	6–12	—
Orange *Flavobacterium*	2–5	4–8	—
Pseudomonas	1–2	1–3	5–10
E. coli	2–3	2–3	—
A. Aerogenes	1–2	2–3	—
A. cloaceae	3	1–2	—

Salt Tolerance in Fish

From 3 to 5 per cent sodium chloride in the fish made either no difference or accelerated the production of spoilage odours. The growth on salted fish muscle differed from that in the nutrient brine, in that the orange cultures grew at 5 but not 8 per cent and the yellow cultures at 8 but not 12 per cent salt. Once again it was observed that by making a heavier initial inoculation, the maximum salt concentration permitting growth could be raised by one or two per cent. The figures given are for the lighter inoculations.

The Significance of Inoculations with Pure Cultures

Tests such as those described above, which are useful for identifying the activities of individual species, have very definite limitations when the results are used for interpreting the spoilage that occurs with the normal mixed microflora. To obtain a truer picture, one must have data showing the relative growth of the different bacteria in the fish under normal commercial conditions as well as the numbers that are initially on the fish.

Zobell and Feltham (1934) observed that from several thousand colonies isolated from plates poured from sea water and marine mud, 31.3 per cent had a yellow pigment and 15.2 per cent were orange. These consisted chiefly of species of *Micrococcus* and *Flavobacterium*. In the present work from over 5500 colonies on the higher dilution plates poured from the skin and surface slime of 31 cod fish, it was found that approximately one-quarter were either orange or yellow colonies. Almost the same proportion was obtained from freshly cut cod fillets.

However, as pointed out in the introduction, the proportion of the organisms of the different genera does not remain constant. For example, in one particular group of similar fillets stored at 2° to 3°C., the progressive change in the proportion of these orange and yellow chromogens to the achromogenic colonies was as follows:

initial	1:9
24 hours	1:13
72 hours	1:66
120 hours	1:950

After the fifth day the chromogens were so outnumbered that they did not show up in any of the plates.

In addition to differences in growth rates, some bacterial species appear to have an inhibiting action on others. For example, similar pieces of sterile fish muscle were cut from fresh cod. These were inoculated as follows: (1) with *Flavobacterium* species only; (2) with *Pseudomonas* species only; (3) with equal numbers of *Flavobacterium* and *Pseudomonas*. They were then stored at approximately 2° to 3°C. Plate counts were made to determine the proportions of each of these genera after 2, 4, 6 and 8 days storage, which were as follows:

Approximate proportions of *Flavobacterium* to *Pseudomonas* colonies

Days storage	Fl. and Ps. on separate samples	Fl. and Ps. on same samples
2	1:1	1:1
4	1:15	1:14
6	1:12	1:252
8	1:56	1:>10,000

In these tests the *Flavobacterium* continued to grow on the fish, but much more slowly than the *Pseudomonas*. Where the separate cultures were on the fish, the *Flavobacterium* multiplied 34-fold during the first six days of storage; where the cultures were mixed the corresponding increase for the same period was only 5-fold.

A somewhat similar observation was made by Tobin, Alford and McCleskey (1941) in canned crab meat held at 1° to 5°C. The initial counts varied from 100,000 to 3,000,000 per gram and consisted principally of Cocci with a few

Bacillus, Achromobacter, Flavobacterium, Aerobacter, Escherichia and *Pseudomonas.* The total count increased steadily during storage and at the end of a week consisted almost wholly of *Pseudomonas* and *Achromobacter.*

Table IV, taken from the work of Wood (1940) shows the changes observed in the proportion of the organisms in the different genera on fish as they move from the seaports to the retail stores.

TABLE IV. Changes in the percentages of the numbers of organisms in fish during various stages of handling (Wood, 1940).

Genus	Ports	Trawlers	Market	Retail
Achromobacter	8	30	18	45
Pseudomonas	0	21	8	27
Flavobacterium	50	10	11	3
Micrococcus	42	30	52	14
Bacillus	0	9	10	10

Anderson (1947), working at this laboratory, picked representative colonies from plates poured daily from fresh cod fillets stored at 3°C. These cultures were identified to genus, and the results are shown in Table V. It can be seen that after six days, over 80 per cent of the bacteria on the fish consist of *Pseudomonas* and *Achromobacter,* while the *Flavobacterium,* which had been 33.4 per cent at two days, was reduced to 3 per cent at six days.

Pivnick (1948), also working at this laboratory, followed the proportion of the various genera of bacteria on unfrozen cod muscle and the changes in the proportion resulting from freezing and storage in the frozen condition. In this work the cod muscle was aseptically cut from the whole fish and all the samples were similarly inoculated by immersing them in a mixed flora obtained from fish slime and fish faeces. He found that freezing the fish muscle for 24 hours caused a decrease of 40 to 60 per cent in the total bacterial population and

TABLE V. Changes in the proportion of *Achromobacter, Pseudomonas* and *Flavobacterium* on cod fillets during storage at 3°C. as shown by the percentage of colonies picked at random from plates poured from the fish on successive days. Also, the trimethylamine value and odour of fillets at time of plating.

Genus	Percentage of colonies 2 days	4 days	6 days	8 days
Achromobacter	17.8	38.0	51.6	47.8
Pseudomonas	9.6	19.2	29.3	35.4
Flavobacterium	33.4	11.9	3.0	3.2
Odour of fillets	fresh	slightly fishy	fishy-putrid	putrid
Trimethylamine value*	0.57	11.1	40.5	39.0

*mg. of Me_3N nitrogen per 100 g. fish.

storage at −12° or −23°C. for three or six months caused a further substantial reduction. During these same treatments the percentage of the *Flavobacterium* in the total bacterial population remained remarkably constant:

	Flavobacterium %
Initial unfrozen fish	43.1
Slow frozen – 24 hours at −12°	40.6
Fast frozen – 24 hours at −23°	46.8
Slow frozen – stored 3 months at −12°	50.9
Fast frozen – stored 3 months at −23°	59.3
Slow frozen – stored 6 months at −12°	48.1
Fast frozen – stored 6 months at −23°	38.5

In a similar experiment the slow-frozen fish after six months storage at −12° was then held at 3°C. for three days. The percentage of *Flavobacterium* dropped to 1.9 per cent while the Gram-negative achromogenic rods (principally *Achromobacter*) increased to 98.1 per cent.

DISCUSSION

Several species of *Flavobacterium* are abundant on fresh fish; they grow at temperatures close to freezing, the majority decompose protein, and many reduce trimethylamine oxide. Therefore, one might easily conclude that they are among the more important of the fish-spoiling bacteria. And undoubtedly if sterile fresh fish was contaminated with a mixed group of *Flavobacterium*, it would develop very offensive odours and might also become discoloured.

But this does not necessarily mean that they contribute significantly to the spoilage under *normal* commercial conditions. Three characteristics determine whether an organism is a potential fish spoiler: its ability (1) to grow rapidly on the fish; (2) to grow readily at low temperatures; and (3) to initiate some chemical change resulting in the production of an offensive odour or flavour. In fresh fish the offensive products most rapidly formed are the result of the activities of respiratory enzymes (volatile acids, trimethylamine, etc.). The proteases, lipases and other hydrolytic enzymes are more active when bacterial cells are undergoing their logarithmic growth phase. Hence, we have a succession of spoilage odours from stale, sweet, fruity and fishy to putrid and faecal types.

From the standpoint of the merchant selling the fish, it is this initial spoilage, the "stale", "sweet", "sour", "fruity" and "fishy" odours, that renders the fish unpalatable and reduces its quality. Once the putrid or faecal stage has been reached, it is no longer fit for consumption. And in most cases, the same organisms that produce the initial "off" flavours are able to bring about the subsequent putrefaction. (Castell and Anderson, 1948).

The results given above have shown that at 25°C., pure cultures of *Pseudomonas* produce off-odours on fish 24 to 48 hours before the worst species of the *Flavobacterium*; at 3°C. this difference is extended from three to five days.

Many members of the *Flavobacterium* produced either no off-odours or very mild types at this temperature.

It has also been shown that the *Pseudomonas* and other achromogenic Gram-negative rods rapidly outgrow *Flavobacterium* on fish and become the predominant microflora by the time spoilage odours become perceptible. Members of *Flavobacterium* are not more sensitive to freezing and storage than other organisms on fresh fish. But once the frozen fish has been thawed out, they are once again rapidly outgrown by the achromogenic Gram-negative rods.

In comparison with other non-halophilic bacteria found on fish, the *Flavobacterium* were not particularly salt tolerant. Cultures of *Pseudomonas* grew faster and produced off-odours on fish at salt concentrations that stopped or greatly inhibited the *Flavobacterium*.

These results would appear to confirm the earlier and more limited observations (Castell and Anderson, 1948) which indicated that Flavobacteria grew more slowly and produced less offensive odours in fish than members of the genera, *Pseudomonas, Achromobacter* and *Proteus*. The possible exception is that some of the strongly proteolytic, deep-orange cultures of *Flavobacterium*, which are slower in developing than the achromogenic species, are certainly not less offensive when they do get a start on the fish.

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Improved Medium for Red Halophilic Bacteria from Salt Fish

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ABSTRACT

The relatively slow development of red halophilic bacteria on laboratory culture media has long been noticed to cause distinct disadvantages in the cultural studies of the types responsible for the reddening of salt fish. Thus, routine analyses of salt and salt fish samples for the presence of red bacteria and also disinfectant studies leading to the elimination of the resulting contamination are seriously delayed. Lochhead's skim milk-salt agar medium has been modified by the addition of $MgSO_4.7H_2O$, 5 g.; $Mg(NO_3)_2.6H_2O$, 1 g.; $FeCl_3.7H_2O$, 0.025 g.; Proteose Peptone No. 3, 5 g.; and glycerol C.P., 10 g. to 1000 ml. of medium. In addition to technical improvements, definite stimulation of growth has been obtained; the incubation time being cut in half and the volume of growth several times increased.

HISTORICAL

Halophilic bacteria that cause reddening of salt fish have been investigated extensively for the last half-century not only on account of their extraordinary physiological and morphological characteristics, but mainly on account of their considerable economic importance. Serious losses suffered through contamination have been reported by Harrison and Kennedy (1922) and Finn (1941) and still continue to cause a deep concern amongst salt fish producers and exporters. It seems that these losses increase at a rate proportional to that of the expanding bulk production of salt fish.

The main efforts of several investigators have been centered along two main courses: isolation, cultivation and identification of halophilic bacteria, and development of methods to eliminate the resulting contamination. At the present time the cultivation of these bacteria still offers many difficulties, while the removal of contamination from salt fish, salt stocks, salt bins and salting vats has not yet been effectively adapted to practical use. For both these problems the long incubation period of halophilic bacteria is a distinct disadvantage.

In his extensive review of the literature dealing with the study of the organisms responsible for the red discoloration of salt fish, Boury (1934) noted the relatively long incubation periods before the appearance of the characteristic colour on agar media. Depending on the strains studied, he reported incubation periods ranging from 6 to 40 days and recognized the necessity for improved media. Similar conclusions were obtained by Harrison and Kennedy (1922) who noticed only slow and moderate growth on the best of the various media tested. Later, Harrison (1923) isolated the causative organisms from "Rusty Herring" on 16 per cent codfish agar and other fish media, but three weeks were necessitated for the appearance of growth.

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In a bacteriological study on the discoloration of salted hides, Lochhead (1934) isolated a variety of organisms including micrococci, sarcinae, spore-forming and non spore-forming rods. For isolation and cultivation studies he devised three culture media; his skim milk-salt agar was found best as growth appeared between three and five days.

Studying "pink" in salted fish, Shewan (1936) tried several media and obtained suitable development on Clayton's (1927) rice medium and on a sulphite agar with saturated salt. Similar studies by Gibbons (1936) were made on media made up of wheat flour and fish broth and later on Lochhead's medium where satisfactory results were obtained. Throughout his investigations on the growth of red halophilic bacteria, Hess (1942a) used advantageously a modification of skim milk-salt agar of Lochhead. However, in all these three separate studies, the time required for cultivation was not mentioned.

For the isolation of halophilic bacteria from environments of low NaCl concentrations such as soil, water and dung, Stuart (1938) first inoculated into an enrichment nutrient brine. After an incubation period of 30 to 90 days, transfers were made unto Lochhead's milk-salt agar where the cultures were kept another 30 days before recording growth. Following this same enrichment technique for the isolation of red halophilic bacteria from sea-water, fish slime and intestines, Hess (1942b) had to allow a minimum period of 30 days before noticing any development.

Therefore it seems to be generally conceded that the cultivation of halophilic bacteria, especially the types responsible for the red discoloration of salt fish, is an inconveniently long operation lasting several days, when grown in most of the media advocated. Consequently, studies dealing with the cultural characteristics of these organisms, the evaluation of bactericidal agents and the bacteriological analysis of salt stocks, salt fish and contaminated areas are seriously hampered by these long incubation periods. This paper deals with the preparation of an improved medium that will promote a heavier growth of red halophilic bacteria in a shorter incubation time.

EXPERIMENTAL

Preparation of Inocula

The inocula used for testing the media were prepared from an actively growing slope culture of a Micrococcus species isolated from reddened codfish. Cultural characteristics and microscopic examination have showed its similarity to "*Sarcina littoralis*" described by Poulsen (1879-80) and probably to "*Micrococcus morrhuae*" described by Klebahn (1918). A heavy suspension of the culture was prepared in a 20 per cent NaCl solution, but sufficiently diluted to remove all trace of pink coloration. This constituted the heavy inoculum. A lighter inoculum was obtained from the former by further diluting one part of the heavy suspension with 100 parts of NaCl solution. The ability of the media to develop red halophilic bacteria was also tested with a brine solution made up of 30 per cent commercial salt that had previously been found con-

taminated with red bacteria. This last method of inoculation was found very appropriate since it is the one used in the laboratory for the bacteriological analysis of salt samples and since red bacteria present in contaminated salt have longer lag periods than actively growing cultures.

Preparation of Medium

Numerous attempts have been made by us towards the final choice of a basic substance around which a more satisfactory medium would be built up. The following have been tried in varying concentrations: cod muscle press juice, hot and cold water extracts of fresh and salted codfish, suspensions of macerated cod muscle, acid hydrolysates of cod muscle, potato extracts, wheat and rice flour pastes, etc. Also, since on several samples of reddened codfish the discoloration had been observed to proliferate more rapidly and more heavily along the remaining section of the vertebral column, extracts were prepared from spinal chords and intravertebral gelatinous material obtained from fresh cod. In most cases where extracts were prepared, tap water, distilled water and seawater were used alternately for the purpose of comparing their respective effects.

None of these substances proved very successful or showed any marked advantage over Lochhead's skim milk-salt agar medium. Skim milk was still the best basic material in the preparation of media for the cultivation of red halophilic bacteria since it supported fair growth and supplied a better background for the evaluation of the extent of development and shade of pigmentation. This explains why Lochhead's medium has been so widely used by recent investigators and why it was chosen in the present work as the basis for the following modifications.

Of a wide series of inorganic salts added to the regular skim milk-salt agar, $Mg(NO_3)_2$, $MgSO_4$ and $FeCl_3$ showed a definite stimulating effect. As shown in Table I the optimum concentrations are: $MgSO_4$, 0.5 per cent; $Mg(NO_3)_2$, 0.1 per cent; and $FeCl_3$, 0.0025 per cent.

Table I. Effect of individual inorganic salts on the growth of red halophilic bacteria in plain skim milk-salt after 5 days at 37°C. (+ = growth on control plates of plain skim milk-salt agar).

Concentration (per cent)	$MgSO_4$	$Mg(NO_3)_2$	$FeCl_3$
1.0	+	+	+
0.5	+ + +	+	+
0.25	+ +	+ +	+
0.1	+	+ + +	+
0.05	+	+ +	+
0.01	+	+	+
0.005	+	+	+
0.0025	+	+	+ +
0.001	+	+	+

The action of a number of organic nitrogen and carbohydrate sources was also investigated for the purpose of determining if any growth-stimulating effect could be obtained by their addition to the basic skim milk-salt medium. The results obtained are shown in Table II. The need for extra organic nitrogen was evidenced by the stimulation caused by the addition of 0.5 per cent Proteose Peptone No. 3 Difco which showed definite advantages over the other Bacto

TABLE II. Effect of various organic nitrogen and carbohydrate sources on the growth of red halophilic bacteria after 5 days at 37°C. (+ = growth on control plates of plain skim milk-salt agar).

Nutrient Source	Concentration 0.1%	0.5%	1.0%
Organic Nitrogen			
1. Bacto-Peptone	+ +	+ +	+ +
2. Proteose Peptone No. 3 Difco	+ + +	+ + + +	+ + + +
3. Bacto-Tryptose	+ +	+ + +	+ +
4. Bacto-Tryptone	+	+ +	+ +
5. Bacto-Asparagine	+	+	+
Carbohydrate			
1. Dextrose	+ +	+ +	+ + +
2. Lactose	+	+	+ +
3. Glycerol	+	+ +	+ + +

products tried. Slightly less stimulation was obtained with tryptose, tryptone and peptone, whereas no improvement was noted by the presence of asparagine. It would seem, therefore, that the latter nitrogen source is not utilized as such by the red halophilic culture and that a more complex combination of nitrogenous substances is required. The inclusion of a carbohydrate source into the medium also proved beneficial as increased development resulted. Dextrose and glycerol in a 1 per cent concentration were found equally superior to lactose. However glycerol was preferred to dextrose because a deeper shade of pigmentation was obtained along with the increase in growth.

The proposed medium used throughout is prepared from a nutrient brine containing $MgSO_4.7H_2O$, 5.0 g.; $Mg(NO_3)_2.6H_2O$, 1.0 g.; $FeCl_3.7H_2O$, 0.025 g.; Proteose Peptone No. 3, 5 g.; glycerol C.P., 10 g.; and C.P. NaCl, 200 g. (which concentration is always expressed in this paper as 20 per cent) in 1000 ml distilled water. This nutrient brine is then divided into two equal portions. To the first, 10 per cent Bacto-skim milk is added and to the second plain Bacto-agar in a 3 per cent concentration. When the agar portion is thoroughly melted and the well suspended skim milk brine lightly warmed, equal amounts of each are carefully mixed together in a flask and 15 to 20 ml. quantities are transferred immediately into petri dishes with the use of a fast-delivery pipette. The use of a pipette instead of pouring from the flask aids in the elimination of air

bubbles produced when the skim milk and agar portions are mixed together. Slopes for the transferring of cultures are prepared in a similar way.

It has been noticed that the medium thus prepared does not have to be sterilized. During the several months the medium was utilized for routine analysis and cultural studies, uninoculated control plates were often incubated but never showed any contamination. It seems that the high NaCl concentration is sufficient to eliminate the most common contaminants. Thus the stock nutrient brine and salt agar can be kept in the cold for several weeks without alteration. However it is advisable to prepare fresh skim milk brine every time it is needed.

Attempts to buffer the medium with the ordinary salts have not yielded any improved results, which led to the conclusion that this operation was unnecessary. The optimum pH of the medium was found to be 7.2-7.5. This range is well in agreement with the findings of Stuart and James (1938a and 1938b) concerning the growth of halophilic bacteria in media of high NaCl concentrations.

Evaluation of Media

Lochhead's codfish medium (Medium B, (1934)), his skim milk-salt medium (Medium C (1934)) and Hess's modification of Lochhead's medium C (1942a) were prepared and inoculated for the purpose of comparing with the improved medium proposed in this paper. The three inocula previously described were tested. Individual drops were transferred by means of a pipette to the surface of the agar media in petri dishes. The plates were removed each day from the incubator kept at 37°C. and carefully examined by visual observation for the time at which red growth first appeared and also for the extent of development and pigmentation. The results obtained from these comparison studies are illustrated in Table III and Figure 1.

Table III. Comparison of media for the time required to evidence beginning of growth of red halophilic bacteria.

Media	Time in days at 37°C. Heavy inoculum	Light inoculum	Contaminated brine
Lochhead's medium B	5	6	10
Lochhead's medium C	4	5	10
Hess's modified medium	4	5	10
Improved medium	2	3	5

DISCUSSION

Figure 1 and Table III show clearly the superiority of the proposed medium over some others that have been advocated and utilized until now. The cultivation of red halophilic bacteria becomes more rapid. With the three inocula

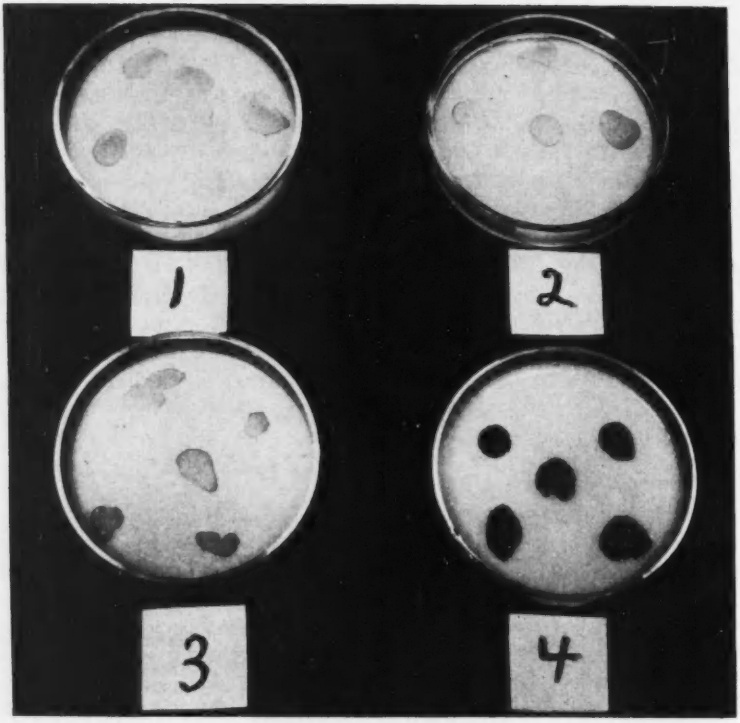

FIGURE 1. Comparison of media for optimum growth of red halophilic bacteria after 6 days at 37°C.: (1) Lochhead's medium C; (2) Lochhead's medium B; (3) Hess's modified medium; (4) improved medium.

tested, development was observed on the improved medium in half the time required by the other media. Also, it yielded a volume of growth several times heavier as evidenced by the thickness of the film and the intensity of pigmentation. These two major improvements contribute greatly to the cultural studies of halophilic bacteria by shortening the usually long and inconvenient lag period. Results of bacteriological analysis of salt samples and salt fish which are always urgently needed, can now be obtained more rapidly.

The requirement for the magnesium ion, both in the sulfate and nitrate form, has been indicated, resulting in a definite activation of growth. However, because of the fact that maximum development of the red halophilic bacteria occurs at different concentrations of the two magnesium salts, 0.5 per cent for the sulfate and 0.1 per cent for the nitrate, it seems that the anions also play a part in growth stimulation. Although to a lesser degree, a trace of ferric chloride produces some stimulation. It has been noticed that the addition of glycerol stimulates pigmentation, changing the light red colour into brick red under identical cultural conditions.

Technical improvements have been noted in the preparation of the proposed medium. With the elimination of the sterilization process, it is now possible to prepare the medium for routine analysis in salt fish plants without excessive care and costly installation. Another advantage is that higher concentrations of NaCl, approaching saturation if required, can be incorporated into the medium. Preliminary observations have shown that the improved medium without skim milk can be advantageously used for the cultivation of associated and unpigmented halophilic bacteria. In the same manner as with the medium containing skim milk, increases in the volume of growth and shortening of the incubation time have been observed.

Work dealing with the preparation of a liquid medium has been initiated. Although cultures of red halophilic bacteria have always shown scanty and slow development in liquid media, definite improvements have been noted. However, when compared to the results obtained with the solid medium, development in liquid media is completely unsatisfactory; the production of the red pigment is appreciably reduced and the volume of growth much smaller. It would appear that red halophilic bacteria, being strict aerobes, can only attain optimum development and pigmentation by surface growth on solid media.

SUMMARY

The elements essential to stimulated growth of halophilic bacteria that cause reddening of salt fish are magnesium sulfate, magnesium nitrate, iron and an organic source of nitrogen, all added to skim milk-salt agar.

Stimulation of growth and increased pigmentation have also been produced by the addition of glycerol.

The proposed medium, modified from Lochhead's skim milk-salt agar, has shown definite improvements in the cultivation of halophilic bacteria resulting in an increased volume of growth and a shortening of the incubation time.

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The Distribution of Vitamin B_{12}-Active Substances in some Marine Invertebrates of British Columbia

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ABSTRACT

Quantitative estimation of the amounts of vitamin B_{12}-active substances in some marine invertebrates was made using a microbiological assay technique. In general, species from phylum Mollusca were found to be richer in these substances than were species from phyla Echinodermata and Annelida and class Crustacea. No definite relationship was found to exist between the content of vitamin B_{12}-active substances in an animal and its phylogeny.

INTRODUCTION

Within the past few years vitamin B_{12} values have been published for a number of biological materials, mainly apropos of mammalian and other vertebrate tissue. Tarr *et al.* (1950) reported the occurrence of vitamin B_{12}-active substances in the organs and flesh of many Pacific coast fishes, also in fishery products including samples of fish meals and fish solubles. They found the organs, especially kidney tissue, of sockeye salmon (*Oncorhynchus nerka*) to be particularly rich in vitamin B_{12}-active substances; a sample lot of the clam *Paphia staminea* was also rich in these substances.

So far there has been no systematic comparative study of the concentration of vitamin B_{12}-active substances in marine invertebrates. If these substances are essential constituents of organisms one would expect to find them in the lower forms of life as well as in the tissue of the more highly organized vertebrates. To test this expectation and to report on the distribution of vitamin B_{12}-active substances in such lower forms as marine invertebrates was the main purpose of this investigation.

MATERIALS

For the microbiological assay of total vitamin B_{12}-active substances all of the specimens were collected alive by the writer. The three phyla of animals chosen as subjects furnished a number of diverse species, most of which are readily available in this British Columbian region. The material was collected chiefly in the Strait of Georgia and the inter-tidal zones of the beaches in the Vancouver area of British Columbia. The specimens were air frozen at about −25°C. within 24 hours or less after capture, and were stored in the dark for not over three months at this temperature until they were examined. Chemically pure materials were used in the preparation of all reagents and media.

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METHODS

I. Sampling

Whenever possible, at least ten individuals of any species were taken as a representative sample of material for assay. Each sample, excepting those with hard calcareous shells, was homogenized in its entirety by means of either a mortar and pestle or a Waring Blendor and a suitable amount was taken for assay. If, however, the animals possessed hard shells of a calcareous nature, only the soft parts of the animal were used. In dealing with animals containing large amounts of fluid as much of the fluid as possible was retained with the sample.

II. Moisture Determination

In order that the results could be expressed on a dry-weight basis, duplicate moisture determinations were made on all samples assayed. Amounts of from 2 to 5 g. of the homogenized material were dried for 24 hours at 100–102°C.

III. Liberation of Vitamin B_{12}-Active Substances

Smith and Parker (1948) resorted to proteolysis to free the vitamin from liver tissue in the original purification of anti-pernicious-anaemia factor. For the present investigation a similar proteolytic procedure was adopted, in which 10-g. portions of each homogenized sample were diluted with approximately 50 ml. of distilled water and the pH was adjusted to 5.0 with 2.5-normal hydrochloric acid. For each gram of material, 0.1 g. of the fungal enzyme, Mylase P, was added to the solution. The enzymatic hydrolysis was allowed to proceed under toluene in an incubator, at a temperature ranging from 47° to 52°C., for 24 hours. Mylase P does not require an activator to enhance the rate of reaction, therefore it was found to be a highly desirable proteolytic biocatalyst. Moreover, Mylase P is almost free of vitamin B_{12} (Southcott, 1950, unpub.). The hydrolysates were stored at about −20°C. in darkness until required. Preliminary tests with raw salmon kidney showed that this hydrolysis gave optimum results in releasing the vitamin B_{12}-active substances.

When ready for use, the hydrolysates were thawed, then diluted to 100 ml. with distilled water and an aliquant was centrifuged. Distilled water was added to the supernatant liquid to give a suitable dilution, usually of the order 1:1000. Using such a dilution each sample was assayed microbiologically in duplicate.

IV. Assay Procedure

The method of Skeggs *et al.* (1950) was chosen for the microbiological assay, the diluted materials being added to the medium prior to sterilization. This method has been used by Tarr (1951) for determining vitamin B_{12} in fermented stickwater (fish press water) and for assay of fish meals (Tarr *et al.*, 1950).

RESULTS

The results of the microbiological assay for vitamin B_{12}-active substances in a number of marine invertebrates, together with relevant data, are given in

TABLE I. Content of vitamin B_{12}-active substances in marine invertebrates.

Phylum	Class and species	Raw tissue pH	Moisture (%)	B_{12}-active substances, mμg. per gram	
				Wet basis	Dry basis
Annelida	Chaetopoda				
	Aphrodite negligens Moore	6.50	84.9	21	140
	**Aphrodite negligens* Moore	6.50	92.6	4	54
	Eudistylia vancouveri (Kinberg)	6.20	86.3	190	1,400
Arthropoda	Crustacea				
	Pagurus hirsutiusculus (Dana)	8.30	66.3	96	290
	Hemigrapsus oregonensis (Dana)	8.45	64.5	49	140
Mollusca	Placophora				
	Mopalia muscosa (Gould)	6.50	86.9	260	1,900
	Gastropoda				
	Pleurophyllidia californica (Cooper)	7.10	90.2	90	920
	An unidentified Nudibranch	7.10	94.8	77	1,500
	Acmaea scutum patina	7.50	81.0	190	990
	Thais lima (Martyn)	8.50	80.8	550	2,900
	Pelecypoda				
	Schizothaerus nuttallii (Conrad)	6.80	85.7	250	1,800
	Paphia staminea Conrad	6.80	83.4	310	1,900
	Mytilus edulis Linnaeus	6.60	86.9	120	930
	Pecten caurinus (Gould)	6.40	87.1	240	1,900
	Ostrea gigas Thunberg	6.30	82.7	100	570
	Ostrea gigas Thunberg (diff. locality)	6.20	82.6	130	740
	Cephalopoda				
	Rossia pacifica Berry	6.65	81.3	130	680
Echinodermata	Asteroidea				
	Mediaster aequalis Stimpson	7.50	72.9	110	410
	Pisaster ochraceus (Brandt)	7.20	70.9	180	600
	Ophiuroidea				
	Ophiura sarsii (Lutken)	8.40	46.9	150	280
	Echinoidea				
	Strongylocentrotus dröbachiensis (Müller)	6.30	89.8	100	1,000
	Lovenia cordiformis A. Agassiz	6.60	77.3	30	130
	Holothuroidea				
	Stichopus californicus (Stimpson)	6.50	93.1	17	250
	** *Cucumaria miniata* (Brandt)	6.75	85.8	12	84
	** *Cucumaria lubrica* Clark				
	Molpadia arenicola (Stimpson)	6.10	91.9	20	250

*The skin or outer covering of this species was not included in the assay.
**These two species were assayed together.

Table I. The work of Tarr *et al.* (1951) has indicated that in fishery materials much of the vitamin occurs as vitamin B_{12a} or chromatographically similar forms, and that separate sterilization of these materials followed by aseptic addition to previously sterilized assay medium usually gives high apparent recovery of the vitamin. However, the values of total vitamin B_{12} activity detected, as reported in Table I, suffice for comparative purposes in that they probably represent the amount of vitamin B_{12} present.

In general, of the species assayed, molluscs contained the largest amounts of vitamin B_{12}-active substances. The crustaceans gave rather lower values than the other groups of animals as a whole. Of the two species of annelids, the sea mouse (*Aphrodite*) had a low concentration of vitamin B_{12} activity, while the tube worm (*Eudistylia*) contained a high concentration. One genus of Echinodermata, namely a sea urchin, *Strongylocentrotus*, contained high concentrations of vitamin B_{12}-active substances, while an exceedingly low concentration was found in another genus, *Cucumaria*.

DISCUSSION

As indicated in Table I, the moisture content of all the species assayed was quite high except for the starfish, which was rich in calcareous material. The pH value for the raw tissue of the animals is shown to range from 6.10 to 8.45, but the variation bears no recognizable relationship to the other data listed.

Although no specific study was directed towards determining the concentration of vitamin B_{12}-active substances in various organs of each species assayed, it is observed (Table I) that *Aphrodite negligens* Moore, when assayed whole, contained about two and a half times the concentration of vitamin B_{12}-active substance as when assayed after the skin had been excluded from the tissue homogenate.

Although no account was taken of age, weight, sex or seasonal variations, it is apparent from the results that striking differences, with respect to the concentration of vitamin B_{12}-active substances, exist between species of the same class and between species of the same phylum. Further, the presence of vitamin B_{12} activity in all of the species examined is additional evidence of the probability that this vitamin is universally present in the animal kingdom.

Nevertheless, as the entire animals including their alimentary tract contents were assayed, it should be kept in mind that some or all of the vitamin B_{12} activity may be due to synthesis in the digestive tract by micro-organisms.

In consideration with the data of researches carried out on echinoderm embryology by Fell (1948), on phosphagen in Echinodermata and Protochordata by Baldwin and Yudkin (1950), and on comparative biochemical studies on the sterols of marine invertebrates by Bergmann (1949), additional data secured in the present investigation displayed certain properties compatible with comparative biochemical aspects of evolution (Maxwell, 1951). All things being considered, however, there is very little evidence in the present results

to indicate any definite relationship between the proportion of vitamin B_{12} in an animal and its position with respect to phylogeny.

To compare marine invertebrate tissue with vertebrate tissue generally, it would seem from the data available that vitamin B_{12} is much more abundant in the former. Most of the vitamin B_{12} values for vertebrate tissue reported on a fat-containing basis by Lewis *et al.* (1949) are very low, there being only two values as high as 500 mμg. per gram, for beef liver and beef kidney respectively.

CONCLUSIONS

Vitamin B_{12}-active substances are present in marine invertebrates in amounts ranging from 84 mμg. to 2,860 mμg. per gram of dry tissue.

There is practically no evidence in the present results to demonstrate that the proportion of vitamin B_{12}-active substances in marine invertebrates bears a relationship to their phylogeny.

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